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Effects of Dibutyl Phthalate in Male Rabbits following *in Utero*, Adolescent, or Postpubertal Exposure

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We evaluated sequelae in male rabbits following exposure to dibutyl phthalate (DBP) at a dose level known to adversely affect testicular function in rodents without causing systemic toxicity. Because rabbits have a relatively long phase of reproductive development simulating better than rodents the reproductive development of humans, and because the use of rabbits facilitates multiple evaluations of mating ability and seminal quality, we used this animal model. Rabbits were exposed to 0 or 400 mg DBP/kg/day *in utero* (gestation days [GD] 15–29) or during adolescence (postnatal weeks [PNW] 4–12), and male offspring were examined at 6, 12, and 25 weeks of age. Another group was exposed after puberty (for 12 weeks) and examined at the conclusion of exposure. The most pronounced reproductive effects were in male rabbits exposed *in utero*. Male offspring in this group exhibited reduction in numbers of ejaculated sperm (down 43%; $p < 0.01$), in weights of testes (at 12 weeks, down 23%; $p < 0.05$) and in accessory sex glands (at 12 and 25 weeks, down 36%; $p < 0.01$ and down 27%; $p < 0.05$, respectively). Serum testosterone levels were down (at 6 weeks, 32%; $p < 0.05$); a slight increase in histological alterations of the testis ($p < 0.05$) and a doubling in the percentage (from 16 to 30%, $p < 0.01$) of abnormal sperm; and 1/17 males manifesting hypospadias, hypoplastic prostate, and cryptorchid testes with carcinoma *in situ*-like cells. In the DBP group exposed during adolescence, basal serum testosterone levels were reduced at 6 weeks ($p < 0.01$) while at 12 weeks, testosterone production *in vivo* failed to respond normally to a GnRH challenge ($p < 0.01$). In addition, weight of accessory sex glands was reduced at 12 weeks but not at 25 weeks after a recovery period; there was a slight increase in the percentage of abnormal sperm in the ejaculate; and 1/11 males was unilaterally cryptorchid. In both of these DBP-treated groups, daily sperm production, epididymal sperm counts, mating ability, and weights of body and nonreproductive organs were unaffected. Thus, DBP induces lesions in the reproductive system of the rabbit, with the intrauterine period being the most sensitive stage of life.

Key Words: **DBP; reproductive toxicity; abnormal male sexual differentiation; ejaculated sperm counts; atypical germ cells; semen quality; testosterone.**

Phthalates, used as plasticizers to produce polymeric materials and a variety of consumer products, are among the highest-volume chemicals produced with an estimated 2–8 million tons per annum (WHO, 1992). Environmental contamination or exposure occurs as a result of the manufacture, use, or disposal by leaching out of plastic products or directly through one of the numerous consumer products (cosmetics, pharmaceuticals, etc.) (Autian, 1973; Jaeger and Rubin, 1972; Perwack *et al*., 1981). One of the phthalates, dibutyl phthalate (DBP), has been found in the sediment, water, air, biota, and even in stored foods (Aurela *et al*., 1999; Bove *et al*., 1978; Giam *et al*., 1980; Petersen and Breindahl, 2000). In a recent study, urine samples from a human reference population of 289 adults contained 7 to 294 ng/ml monobutyl phthalate reflecting exposure to DBP (Blount *et al*., 2000). Furthermore, serum concentrations of DBP ranging from 15 to 276 mg/l were detected in 28 of 41 (68%) of females (6 months to 8 years of age) diagnosed with premature thelarche (a physical change characteristic of puberty before age 8) versus none in controls (Colon *et al*., 2000).

DBP is known to be a developmental and reproductive toxicant. In rodents, teratogenic response to DBP was observed only at maternally toxic doses (~1 g DBP/kg/day) from gestation day (GD) 6 to 15 (Ema *et al*., 1993, 1994). When treatment was extended to include the critical window of reproductive development (GD 12–21; GD 14–PND 3; or GD 16–19) marked effects on development of the male reproductive system were observed in rats at 250–750 mg DBP/kg/day. The sequelae included: reduced anogenital distance, retained thoracic nipples, cryptorchidism, hypospadias, malformed epididymis and vas deferens, hemorrhagic testes, and testicular atrophy (Gray *et al*., 1999, 2001; Mylchreest *et al*., 1998, 1999, 2000, 2002). Exposure after weaning, delayed puberty in the male rat, and reduced weights of testis and other reproductive organs (Gray *et al*., 1999). Postpubertal exposure (2 g DBP/

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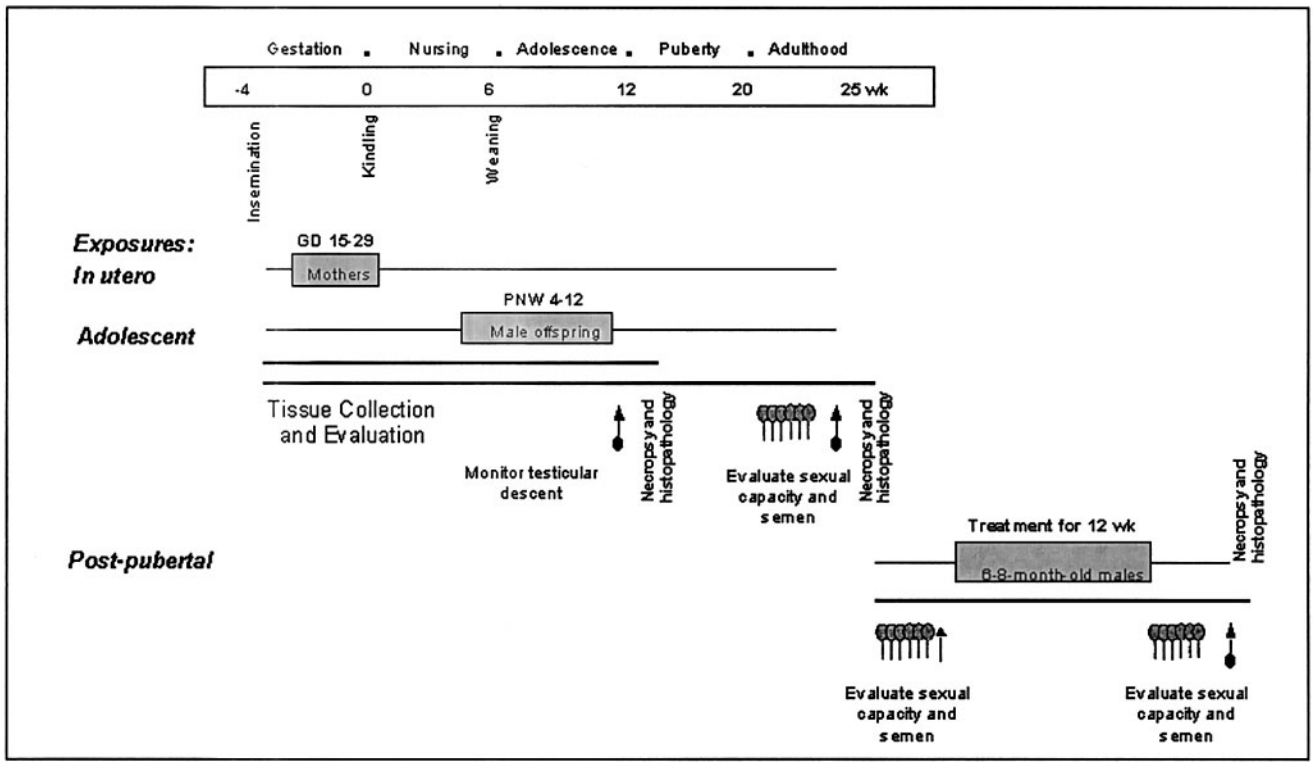

FIG. 1. Overview of experimental design. Arrows indicate hormonal analyses; arrows with a sphere indicate evaluations before and after a challenge with gonadotropin-releasing hormone. GD: gestation day; PNW: postnatal week.

kg/day for 4 to 9 days) also caused a decrease in weights of testes and severe atrophy of seminiferous epithelium in rats (Cater *et al*., 1977; Foster *et al*., 1980; Gray *et al*., 1982).

Although considerable attention has been given to the study of teratological and reproductive toxicity of DBP in rodents, very little is known regarding its effects in species such as rabbits, which have a relatively long infantile (quiescent) period of reproductive development. For example, during late gestation to postnatal week (PNW) 6, the seminiferous epithelium contains only gonocytes, pre-spermatogonia, and Sertoli cells (Gondos *et al*., 1973). From PNW 5 to 7 Leydig and Sertoli cells undergo differentiation, and the onset of spermatogenesis begins around PNW 7–8 when the first adult spermatogonia (types A and B) are formed (Gondos *et al*., 1973, 1976). Spermatocytes are observed at PNW 8, spermatids appear by PNW 12, coinciding with the formation of the tubular lumen, and mature spermatozoa appear in the lumen by PNW 13–14 (Gondos *et al*., 1973). This adolescent period of reproductive development in rabbits, relative to lifespan, is longer than that of rodents and better approximates the comparable phase of human reproductive development. Furthermore, use of rabbit facilitates multiple evaluations of endocrine profiles, mating ability, and semen quality. Use of this species belonging to an order of mammals (lagomorpha) different from that of rodents (rodentia), also addresses the issue that phthalate-induced effects on reproductive development and function may only be rodent-specific. Herein, we report sequelae in male rabbits following exposure to DBP *in utero*, during adolescence, or after puberty at a dose level known to adversely affect testicular function in rodents without causing systemic toxicity.

MATERIALS AND METHODS

Experimental design. Three exposures were performed: *in utero* (GD 15–29), adolescent (PNW 4–12), and postpubertal (12 weeks of exposure) (Fig. 1). *In utero* exposure was begun on GD 15 to ensure that major organogenesis was completed before the start of the treatment and that the window of exposure corresponded with the onset of gonadal differentiation (Gondos and Conner, 1973) and development of the male reproductive tract. Adolescent exposure encompassed the infantile period of testicular differentiation—differentiation of Leydig cells and Sertoli cells, onset of spermatocytogenesis, and initiation of puberty (Gondos *et al*., 1973, 1976). Postpubertal exposure encompassed at least six cycles of the seminiferous epithelium, which has been suggested to be optimal to evaluate the effects of xenobiotics on gametogenesis in adult rabbits (Amann, 1982).

Rabbit husbandry. Six-month-old, specific-pathogen-free, Dutch-Belted rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN) and individually housed in standard stainless steel cages in the CSU animal care facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animals were treated according to the guidelines of the institutional Animal Care and Use Committee. The room was maintained at a 12-h light-dark cycle at approximately 19–21°C and 40% humidity. Rabbits were fed certified rabbit ration (#7009, Harlan, Teklad,

Madison, WI, provided water *ad libitum*, and bedded with kiln-dried aspen shavings ~2 inches below the stainless-steel floor.

After acclimatization for 3–4 weeks, rabbit does were artificially inseminated with 20×10^6 spermatozoa, pooled from semen collected from 10 bucks using the procedures previously described (Veeramachaneni, *et al*., 2001a). Rabbits were palpated for pregnancy 14 days after insemination and pregnant does were randomly assigned to treatment groups for appropriate exposures. On GD 28, pregnant does were provided with nesting boxes. At PNW 3, female pups were culled, and at PNW 6 male pups were weaned and individually housed.

Dosing. Dibutyl phthalate (DBP; CAS# 84–74–2, lot # 03912 ES, 99.8% pure) was obtained from Aldrich Chemical Co. (Milwaukee, WI). DBP was administered orally in a mixture of deionized water:Karo light corn syrup (60:40). For *in utero* exposure, rabbit does were dosed with vehicle only (control; $n = 5$) or 400 mg DBP/kg/day ($n = 8$) from GD 15 to 29. This resulted in 12 control male pups (representing 5 litters) and 17 DBP-exposed male pups (representing 8 litters). In adolescent exposure, rabbit pups ($n = 11$ representing 4 litters) were individually administered 400 mg DBP/kg/day from PNW 4 to 12. Of these experimental animals, one pup from a litter was randomly assigned to a 25-week group (6 each for *in utero* and adolescent exposures) and the remaining to a 12-week group (11 for *in utero* and 5 for adolescent exposures). Because of limited resources, it was not possible to monitor or evaluate all of the male rabbits from each litter until 25 weeks. Likewise, in order to minimize costs and use of animals, control rabbits from *in utero* exposure (6 each for 12- and 25-week age groups) were utilized as controls for the adolescent exposure experiment. Given the observations seen herein, follow-up studies are being designed to replicate and expand understanding of the adverse effects of DBP on the male reproductive system.

For postpubertal exposure, 6–8-month-old bucks ($n = 12$) were trained to ejaculate into an artificial vagina. Three semen samples were collected on alternate days and analyzed prior to the beginning of treatment. Rabbits were ranked based on body weight and before-treatment seminal parameters and alternately assigned to 0 ($n = 6$) or 400 mg DBP/kg/day ($n = 6$), ensuring representative distribution between the two treatment groups. Treatment continued for 12 weeks.

The chosen dose of DBP was based on previous reports that adverse effects on male reproductive development occur between 100 and 500 mg DBP/kg/day without maternal or systemic toxicity (Foster *et al*., 2001; Mylchreest *et al*., 1998, 1999, 2000).

General procedures. For in utero and adolescent exposure groups, body weights were recorded at 6, 12, and 25 weeks of life. Testicular descent was monitored weekly from 6 weeks of life by scrotal palpation until descent was complete, or 12 weeks of age. At 6, 12, and 25 weeks, 4 to 5 ml of blood was collected by jugular puncture, and serum separated and stored at –20°C for hormone assays.

For postpubertal exposure, body weights were recorded before treatment and weekly thereafter. Serum samples were collected before and at the conclusion of treatment and were stored as described above.

Evaluation of mating ability and semen characteristics. Mating ability and semen characteristics were evaluated according to procedures previously described (Veeramachaneni *et al*., 2001a). Briefly, male rabbits were trained to ejaculate into an artificial vagina using a female teaser, and one seminal ejaculate was collected every third day between 22 and 24 weeks of age ($n = 6$ ejaculates/animal) for *in utero* and adolescent exposure groups. For postpubertal exposure, three ejaculates (per animal) were collected before the beginning of the treatment and three after the end of treatment.

For each episode when an ejaculate was collected, mating ability was subjectively evaluated by monitoring the outcome and recording: (1) sexual interest, (2) status of penile erection, (3) number of mounts to accomplish ejaculation, and (4) time taken to ejaculate after the introduction of a female teaser. Once the teaser was introduced, a maximum period of 180 s was allowed for evaluation.

For each sample, the ejaculate volume (after removing the gel) was recorded (to the nearest 0.05 μl) and an aliquot (50 μl) of semen was fixed in 950 μl of buffered formal saline and stored at 4°C until evaluation. Sperm concentration was determined by hemocytometer and total sperm per ejaculate calculated by multiplying the value of concentration of sperm by the corresponding ejaculate volume. Morphologic features of sperm were evaluated in a treatment-blinded manner, using a light microscope equipped with differential interference contrast optics. Two hundred sperm/ejaculate were evaluated in wet smears for abnormalities of the acrosome, head, mid-, and principal pieces, retention of cytoplasmic droplets, and presence of residual cytoplasm using criteria previously established for rabbits (Veeramachaneni *et al*., 2001a).

Tissue sampling. Rabbits were euthanized by CO_2 asphyxiation at 12 or 25 weeks of age for *in utero* and adolescent exposures and at the end of a 12-week postpubertal exposure (10–12 months of age). Liver, kidney, thyroid, testes, epididymides, accessory sex glands, and hypothalamus were evaluated for any gross abnormalities, removed, and weighed. Testes and epididymides were weighed individually. In the 12-week group, both left and right testes, and in the 25-week group, only left testes were sliced into two pieces; one piece was fixed in Bouin's fixative and the other in 4% glutaraldehyde in 0.1 M sodium cacodylate and processed for light and transmission electron microscopy (Veeramachaneni *et al*., 1993). The right testis was decapsulated, weighed, frozen, and stored at –80°C to determine the daily sperm production (DSP). The right epididymis was dissected from the testis, cut into two segments (caput-corpus and cauda), weighed, frozen, and stored at –80°C to determine the epididymal sperm reserves (ESR). The hypothalamus was removed by incisions rostral to the preoptic area, caudal to the mamillary bodies, lateral to the hypothalamic sulci, and dorsal to the anterior commissure. The hypothalamus was bisected through the infundibulum; one-half was weighed, placed in formic acid:methanol (1:9, v/v), and stored at –20°C until assayed for gonadotropin-releasing hormone (GnRH; Nett and Adams, 1977).

Daily sperm production and epididymal sperm reserves. Daily sperm production and epididymal sperm reserves were determined by procedures described for rabbit (Amann and Lambiase, 1969) with minor modifications. Briefly, testicular parenchyma was thawed, minced on a watch glass, and homogenized for 1 min in a semi-micro Waring blender using 50 ml buffer (0.145 M NaCl containing 4 mM NaN_3 and 0.05% [v/v] Triton X-100). The number of homogenization-resistant elongated spermatids was determined without further dilution, using a hemocytometer. DSP was calculated by using a time divisor of 5.35 days (Amann et al., 1974) and expressed per weight of testicular parenchyma. Capita-corpora and caudae epididymidis were thawed, minced on a watch glass, homogenized for 3 min using 50 and 125 ml of buffer, and counted using a hemocytometer without further dilution. ESR was expressed per epididymal segment.

Histopathology of the testis. Seminiferous epithelium and interstitium were evaluated using 5-μm-thick sections stained with hematoxylin and eosin (light microscopy) and 60–80-nm sections stained with uranyl acetate and lead citrate (transmission electron microscopy). Using a Nikon FXA light microscope, one hundred randomly selected, essentially round ($<1.5 \times$ width) seminiferous tubules from each animal were classified into one of eight different grades, using criteria established for evaluating bovine testis (Veeramachaneni *et al*., 1986), with minor modifications for rabbits. Briefly, grade 0, normal intact seminiferous epithelium; grade 1, seminiferous epithelium with pyknotic cells and desquamation or focal vacuolation; grade 2, seminiferous epithelium intermediate between grades 1 and 3; grade 3, seminiferous epithelium with pre-meiotic germ cells and Sertoli cells; grade 4, Sertoli cells only; grade 5, no seminiferous epithelium, leaving only the basement membrane; grade 6, seminiferous tubule with sperm stasis, sperm granuloma, or mineralization; and grade 7, fibrotic seminiferous tubule. A weight between 0 and 1, based on relative degree of loss of germ cells, was assigned to each grade: 0, 1/4, 2/4, 3/4 to grades 0, 1, 2, 3, respectively, and 4/4 to grades 4 through 7, to indicate relative degree of germinal epithelial loss (DGEL). DGEL was calculated by multiplying the percentage of tubules in each grade by the respective assigned weight and summing products.

In 3 DBP-treated rabbits, one from each exposure group, unique lesions were encountered at light microscopic level. Corresponding tissue sections from these animals, along with six appropriate control sections, were examined

using a JEOL 1200EX transmission electron microscope. Evaluation was limited to characterization of subtle lesions and abnormal cells because quantitative analysis is impractical with electron microscopy.

Hormone measurements. Hypothalamic content of GnRH was determined at 12 and 25 weeks in *in utero* and adolescent exposures and after 12 weeks of postpubertal exposure. Each hypothalamus was homogenized in its storage medium and GnRH was extracted and assayed using established procedures (Moss *et al*., 1980; Nett and Adams, 1977). Samples were analyzed in one batch, and the intra-assay coefficient of variation was 3.2%. Concentrations of testosterone were determined at 6 weeks for *in utero* and adolescent exposures.

To assess the ability of the pituitary-gonadal axis to respond to GnRH input, GnRH-challenge tests were conducted at 12 and 25 weeks on *in utero* and adolescent exposure groups; rabbits in the 25-week age group were not subjected to GnRH challenge at 12 weeks. In postpubertal exposure, concentrations of testosterone were determined before treatment and GnRH-challenge tests were conducted after 12 weeks of postpubertal exposure. Each time, a baseline blood sample was taken, 10 μg GnRH was injected (im), and two samples were collected at 30- and 120-min postinjection. Each sample was extracted and assayed for testosterone using a validated RIA (Berndtson *et al*., 1974). Intra-assay and inter-assay coefficients of variation were 5.6 and 7.5%, respectively.

Statistical analyses. Data were analyzed using Statview (version 5.0, SAS Institute, Inc., Cary, NC). Treatment was fixed for each experiment and all parameters were random. Difference between means was determined by t-test. The level of significance was set at $p < 0.05$. This model was used to evaluate body weights, hormone concentrations, and necropsy data. In postpubertal exposure, data for repeated measures on the same animal (endocrine parameters, sexual capacity, and semen characteristics) were analyzed by comparing before- and after-treatment values. Percentage values were transformed using arcsine of the square root of the percentage/100 to account for any inequalities in variance.

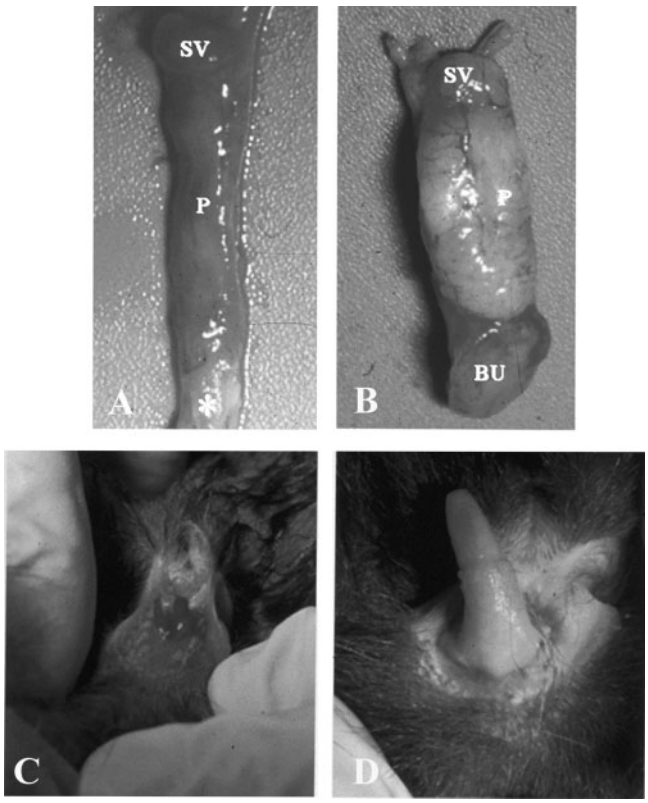

FIG. 2. Accessory sex glands and external genitalia of a 12-week-old cryptorchid rabbit exposed to DBP *in utero* (A, C) compared to an age-matched control (B, D). Note hypoplastic seminal vesicle (SV) and prostate (P) and missing bulbourethral gland (asterisks) (A) in the DBP-exposed rabbit. The external genitalia appear feminized with poorly developed penis and malformed prepuce (C).

RESULTS

There was no effect of DBP treatment on maternal survival or maintenance of pregnancy to full term when administered at 400 mg/kg/day between GD 15 and 29.

Gross Morphological Abnormalities

Descent of testes and differentiation of reproductive tract. Testicular descent occurred between PNW 6 and 9 in all (12/12) controls and in 16 out of 17 pups exposed to DBP *in utero*. The one DBP-exposed rabbit in which testes failed to descend even by 12 weeks also had a poorly developed penis and malformed prepuce (giving an appearance of feminized external genitalia), hypospadias, hypoplastic seminal vesicle and prostate, and agenesis of bulbourethral gland (Fig. 2). However, the epididymides did not manifest agenesis or segmental aplasia. One out of 11 pups exposed to DBP during adolescence was unilaterally cryptorchid.

Body and organ weights. *In utero* exposure to DBP had no adverse effects on F1-male body weights at 6, 12, or 25 weeks of age (Table 1). At 12 weeks of age, weights of paired testes ($p < 0.05$) and accessory sex glands ($p < 0.01$) were lower in DBP-treated pups compared to controls. At 25 weeks, weight of accessory sex glands ($p < 0.01$), but not that of testes ($p > 0.1$), remained lower in DBP-treated rabbits. There were no differences ($p > 0.1$) in weights of paired epididymides at 12 or 25 weeks.

Similar to *in utero* exposure, exposure to DBP during adolescence had no effect on weight gain from PNW 4 to 12 (data not presented) or body weight at 6, 12, or 25 weeks of age. There were no significant differences ($p > 0.1$) in weights of paired testes or epididymides, but the weight of accessory sex glands was lower ($p < 0.05$) in DBP-treated pups at 12 weeks. This decrease in the weight of accessory sex glands was not apparent at 25 weeks.

Postpubertal exposure to DBP also had no effect on body weight throughout the dosing period (data not presented). After 12 weeks of exposure, weights of reproductive and visceral organs did not differ ($p > 0.1$) between DBP-treated and control rabbits (Table 1) but there was a significant increase ($p < 0.05$) in the weight of the thyroid gland in DBP-treated rabbits.

Mating ability. None of the three windows of exposure to DBP had any significant effect on mating ability (data not presented). Failure to ejaculate during 180 s in the presence of a teaser female occurred only once in *in utero* exposure groups

TABLE 1
Effects of *in Utero*, Adolescent or Postpubertal Exposures to 400 mg DBP/kg/day on Organ Weights

	In utero and adolescent exposures						Postpubertal exposure	
	12 wk			25 wk				
	Control (n = 6)	*In utero* (n = 11)	Adolescent (n = 5)	Control (n = 6)	*In utero* (n = 6)	Adolescent (n = 6)	Control (n = 6)	DBP (n = 6)
Body (kg)	1.61 ± 0.06	1.54 ± 0.08	1.54 ± 0.06	2.11 ± 0.05	2.04 ± 0.08	2.04 ± 0.08	2.08 ± 0.07	1.99 ± 0.05
Liver (g)	—	—	—	64.45 ± 3.02	54.96 ± 4.24	62.67 ± 4.67	47.84 ± 2.59	45.90 ± 2.89
Kidneys (g)	—	—	—	14.30 ± 0.61	13.52 ± 0.82	14.06 ± 0.82	12.78 ± 0.36	12.78 ± 0.63
Thyroid (g)	—	—	—	0.16 ± 0.02	0.19 ± 0.03	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01*
Paired testes (g)[a]	2.28 ± 0.15	1.76 ± 0.17*	1.82 ± 0.16	4.25 ± 0.37	4.32 ± 0.21	4.74 ± 0.50	4.57 ± 0.26	4.52 ± 0.27
Epididymides (g)[a]	0.90 ± 0.05	0.79 ± 0.61	0.86 ± 0.09	1.44 ± 0.04	1.36 ± 0.06	1.65 ± 0.13	1.72 ± 0.09	1.65 ± 0.10
Accessory sex glands (g)[a]	1.81 ± 0.18	1.14 ± 0.12**	1.22 ± 0.18*	3.52 ± 0.17	2.54 ± 0.20**	3.85 ± 0.41	3.31 ± 0.32	3.26 ± 0.42

Note. Values represent mean ± SEM.
[a] Data do not include undescended testes or malformed accessory sex glands.
* $p < 0.05$ and ** $p < 0.01$ using *t*-test.

and once in postpubertal DBP exposure groups. These incidences of failure accounted for only 3% (1/36) for *in utero* and 6% (1/18) for postpubertal exposure of the total number of times mating ability was evaluated. In both instances of failure to ejaculate, the rabbits expressed no sexual interest and achieved no erection.

Production of spermatozoa. There were no differences ($p > 0.1$) in DSP and the number of spermatozoa in the caput-corpus or cauda epididymides between control and DBP rabbits in any of the three treatment regimens (Table 2). However, ejaculate volume ($p < 0.01$), sperm concentration ($p < 0.05$), and total sperm per ejaculate ($p < 0.01$) were lower

TABLE 2
Effects of *in Utero*, Adolescent or Postpubertal Exposures to 400 mg DBP/kg/day on Sperm Production and Semen Characteristics

				Postpubertal exposure			
	In utero and adolescent exposures			Control (n = 6)		DBP (n = 6)	
	Control (n =6)	*In utero* (n = 6)	Adolescent (n = 6)	Before treatment	After treatment	Before treatment	After treatment
Tissue homogenates							
Daily sperm production ($\times 10^6$/g testis)	14.65 ± 2.46	16.98 ± 1.17	21.98 ± 2.29	—	23.89 ± 0.99	—	22.85 ± 0.74
Caput epididymal sperm reserve ($\times 10^6$)	139.87 ± 25.21	128.86 ± 13.26	116.53 ± 17.14	—	117.36 ± 11.50	—	116.94 ± 9.21
Cauda epididymal sperm reserve ($\times 10^6$)	407.72 ± 65.31	469.86 ± 39.40	499.15 ± 37.91	—	469.50 ± 51.35	—	449.33 ± 72.33
Semen characteristics							
Ejaculate volume (ml)	0.40 ± 0.03	0.28 ± 0.02**	0.45 ± 0.02	0.56 ± 0.04	0.61 ± 0.04	0.50 ± 0.03	0.51 ± 0.05
Sperm concentration ($\times 10^6$/ml)	563.59 ± 50.33	414.60 ± 28.90*	444.09 ± 36.49	442.71 ± 64.20	523.68 ± 67.09	552.99 ± 63.70	497.89 ± 45.11
Total sperm/ejaculate ($\times 10^6$)	214.74 ± 22.23	121.59 ± 12.08**	203.20 ± 18.18	252.87 ± 40.14	327.33 ± 59.23	282.81 ± 37.24	250.75 ± 27.05
Morphologically normal sperm (%)	83.44 ± 0.76	69.26 ± 1.49**	74.18 ± 0.90**	69.44 ± 3.69	71.06 ± 3.98	80.03 ± 1.2	75.25 ± 1.68*
Acrosome-nuclear defects (%)	6.3 ± 0.5	15.5 ± 0.9**	17.2 ± 1.2**	19.4 ± 3.7	18.3 ± 3.7	9.5 ± 0.9	9.0 ± 1.3

Note. For semen parameters, data from 6 ejaculates (*in utero* and adolescent exposures) or 3 ejaculates (postpubertal exposure) were pooled. Values represent mean ± SEM.
* $p < 0.05$ and ** $p < 0.01$ using *t*-test.

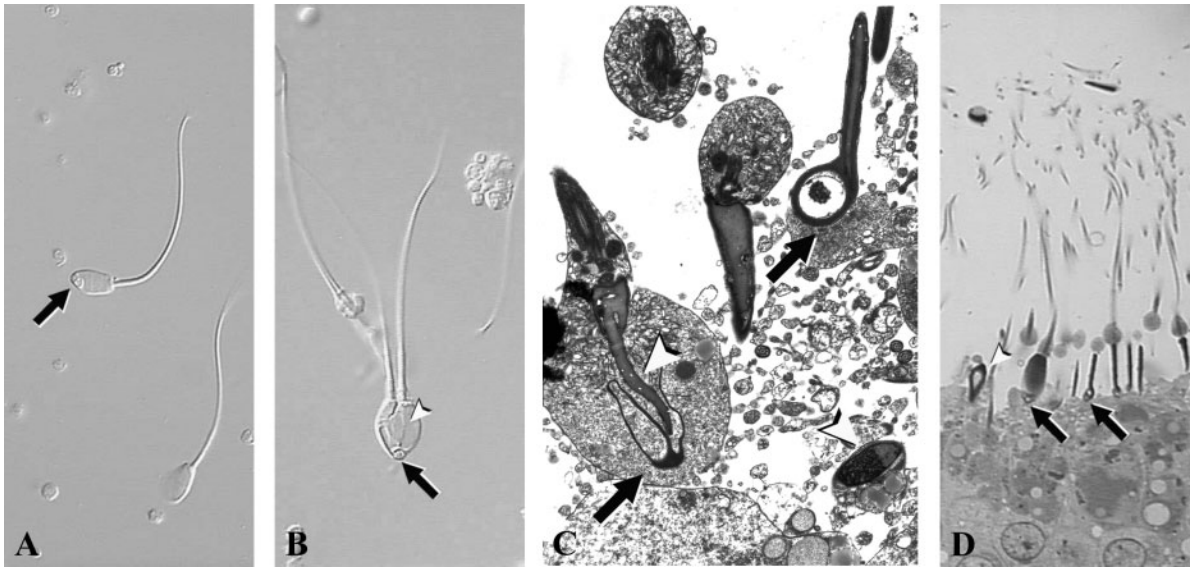

FIG. 3. Sperm acrosomal and nuclear defects in rabbits exposed to DBP *in utero*, during adolescence, or after puberty. Differential interference contrast (A, B), and electron microscopy (C) revealed that these defects included dysplasia and vesiculation of acrosomes and incomplete condensation and vacuolation of sperm nuclei. That these defects originated during spermiogenesis, and not during epididymal transit, is evident from histological examination of testicular sections (D). Arrows: acrosomal vesiculation, Arrowheads: incomplete nuclear condensation. Original magnification (A) ×930; (B, D) ×1030; (C) ×5000.

in rabbits exposed to DBP *in utero* compared to controls. No decline in semen parameters was observed in adolescent or postpubertal exposure groups.

Semen quality, as indicated by sperm morphology, was poorer ($p < 0.01$) in *in utero* and adolescent exposure groups than in controls. DBP-treated rabbits in these two groups had almost twice as many morphologically abnormal sperm (30.1% in *in utero* exposure and 25.7% in adolescent exposure) as controls (16.6%; Table 2). A majority of the abnormal sperm (16 and 18%) had acrosomal and nuclear defects (Fig. 3). These included acrosomal dysplasia and vesiculation and malformed nuclei. A significant reduction (5%; $p < 0.01$) in the number of morphologically normal sperm was also observed following DBP treatment in the postpubertal group. In contrast, control rabbits in this exposure group had a 2% increase in the number of morphologically normal sperm (Table 2).

Histopathology of the testis. Histopathological changes representative of grades 1 and 2 were observed in all rabbits from each treatment group (Table 3, Figs. 4A and 4B). However, the frequency of these changes was higher in DBP-treated rabbits compared to controls. The incidence of grade 4 tubules with total germinal epithelial loss was low, only occurring in a few isolated DBP-treated rabbits from *in utero* and adolescent exposure groups (Fig. 4C). Grades 5–7 tubules were not observed. Reflective of the increased incidence of grades 1 and 2

TABLE 3
Histopathological Changes in the Seminiferous Epithelium after *in Utero,* Adolescent or Postpubertal Exposures to 400 mg DBP/kg/day

	Percentage of seminiferous tubules graded as:							
	n	0	1	2	3	4	5–7	Degree of germinal epithelial loss[a]
In utero and adolescent exposures								
Control	6	88.0	9.2	2.7	—	—	—	3.6 ± 0.2
In utero	6	79.7	14.8	5.0	0.2	0.3	—	6.7 ± 1.0*
Adolescent	6	81.0	15.7	4.2	—	0.2	—	6.2 ± 1.0*
Postpubertal exposure								
Control	6	86.2	9.8	2.2	—	—	—	3.5 ± 0.3
DBP	6	81.2	16.5	2.2	0.2	—	—	5.3 ± 0.7*

[a] Values represent mean ± SEM.
* $p < 0.05$ and ** $p < 0.01$ using *t*-test.

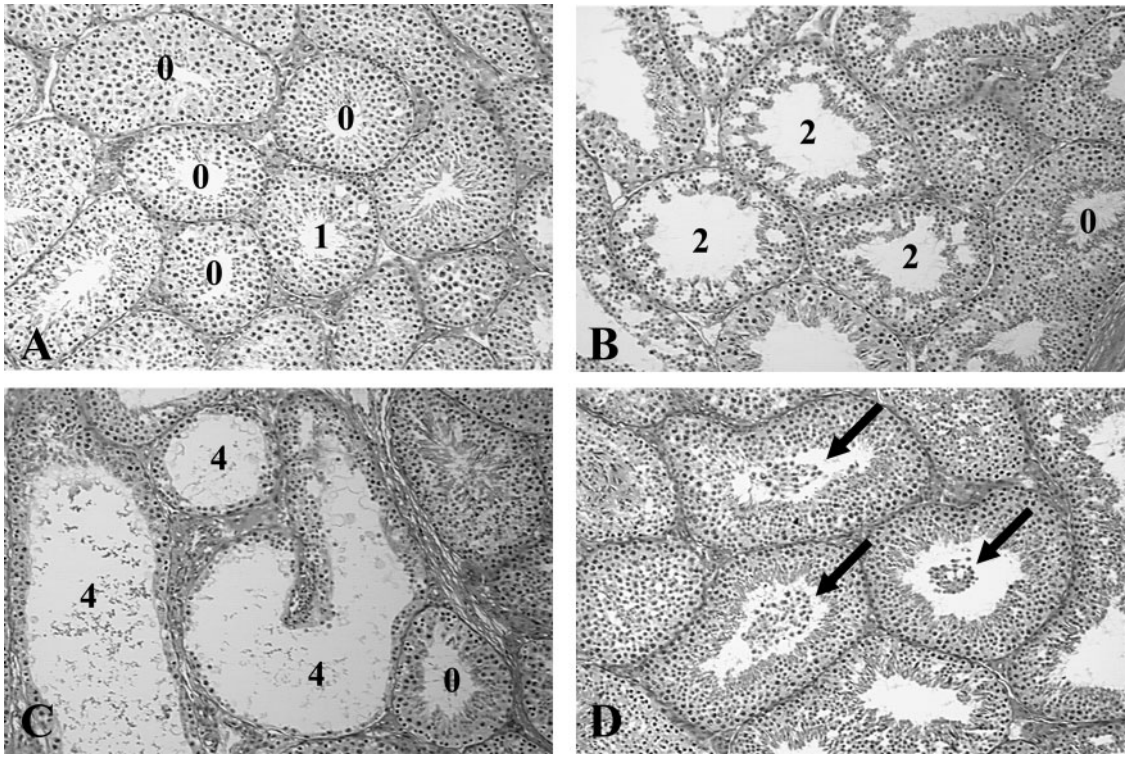

FIG. 4. Pathological changes of the seminiferous epithelium after *in utero*, adolescent, or postpubertal exposure to DBP. Grades 1 and 2 with focal vacuolation of seminiferous epithelium were less frequent in control rabbits (A) than in DBP-treated rabbits (B). Grade 4 tubules containing predominantly Sertoli cells and a few germ cells were observed in rabbits exposed to DBP *in utero* or during adolescence (C). Immature germ cells resulting from premature desquamation (arrows) were seen in the lumina of seminiferous tubules in DBP-treated rabbits (D). Numbers indicate grades of pathological change. Original magnification: ×100; H&E staining.

in DBP-treated rabbits, the DGEL was significantly higher ($p < 0.05$) in all DBP groups than in controls (Table 3). Accordingly, desquamated premature germ cells were frequently observed in the lumina of seminiferous tubules (Fig. 4D).

Atypical germ cells resembling gonocytes and pre-spermatogonia were also encountered in the undescended testes of one 12-week-old, bilaterally cryptorchid rabbit from the *in utero* exposure group (1/17) and one 25-week-old unilaterally cryptorchid rabbit from the adolescent exposure group (1/11). Gonocytes should not be present beyond the first few weeks of life and their presence at 12 and 25 weeks of age indicates an abnormality (Veeramachaneni, 2000a). This germ cell atypia was further characterized using transmission electron microscopy, which confirmed abnormal cells resembling gonocytes and pre-spermatogonia (Fig. 5). The cellular atypia ranged from alterations in the nucleolonema to more advanced changes characterized by unusual membranous profiles, irregular nuclear contours, chromatin clumps, and swollen mitochondria. These morphological features are similar to carcinoma *in situ* (CIS) reported in humans (Nielsen *et al.,* 1974; Skakkebaek, 1972).

Interestingly, atypical germ cells also were identified in the seminiferous epithelium of one rabbit (1/6) in the postpubertal exposure group (Fig. 6). These cells had irregular nuclear contours, perinuclear inclusions, and occasional pale staining of the cytoplasm. Electron microscopic evaluation confirmed light microscopic observations and further revealed pars amorpha in nucleoli, chromatin clumping, and swollen mitochondria.

GnRH and testosterone. There were no changes in the hypothalamic GnRH content ($p > 0.1$) in *in utero* or postpubertal DBP exposures (Table 4), but it was significantly higher ($p < 0.01$) immediately following the adolescent exposure at 12 weeks. This increase in GnRH content was not observed at 25 weeks of age.

Serum testosterone was significantly lower in DBP-treated rabbits at 6 weeks for *in utero* (5.62 ± 0.87 ng/ml; $p < 0.05$) and adolescent (5.03 ± 0.61; $p < 0.01$) exposures compared to controls (8.31 ± 0.84). This decrease was not apparent in subsequent measurements at 12 and 25 weeks. There was no change ($p > 0.1$) in serum concentration of testosterone at the end of treatment in postpubertal exposure compared to before-treatment values.

Injection of GnRH resulted in an increase in serum concentration of testosterone, 30 or 120 min later, in all rabbits exposed *in utero* at 12 or 25 weeks (Table 4). The magnitude

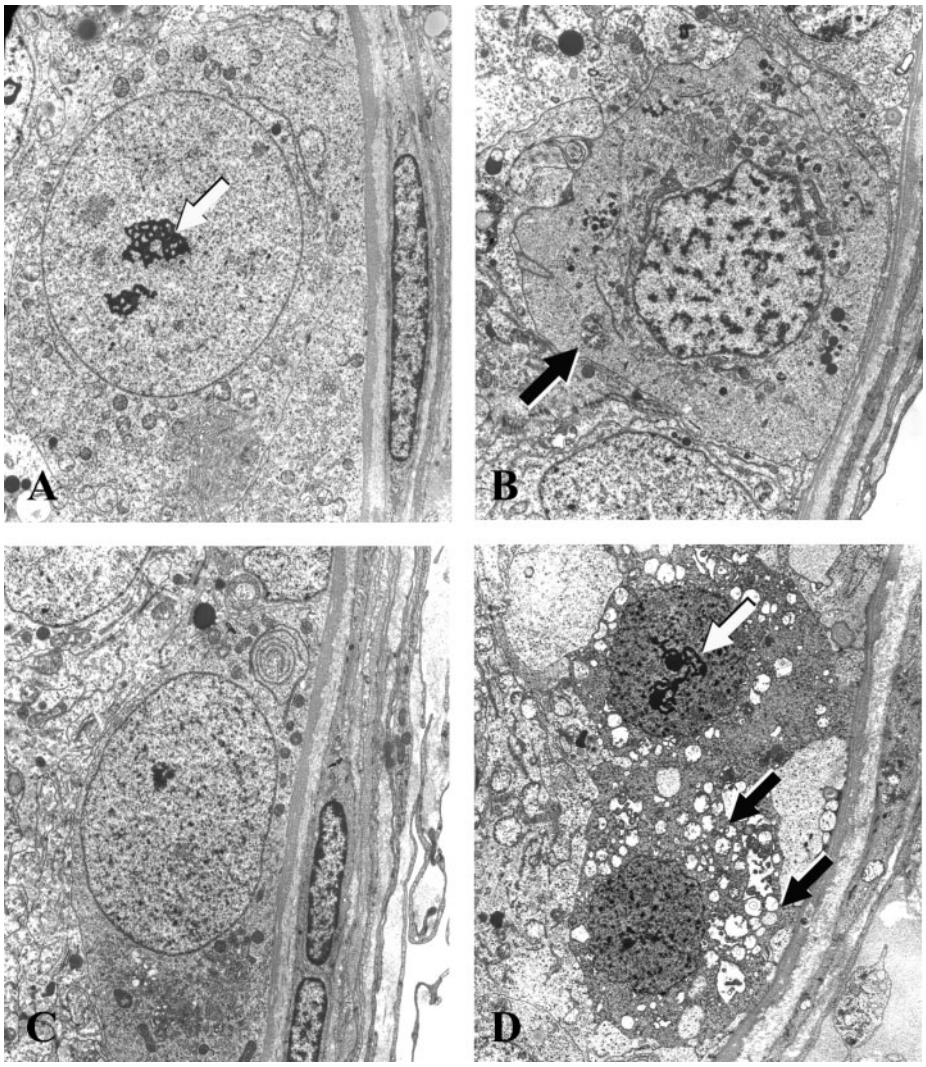

FIG. 5. Transmission electron micrographs of atypical gonocytes (A, B), an unaffected pre-spermatogonium (C), and two atypical germ cells (D) in the undescended testis of a 25-week-old unilaterally cryptorchid rabbit exposed to DBP from PNW 4 to 12. Cellular atypia ranged from alterations in the nucleolonema (white arrow; A, D) to more advanced changes characterized by irregular nuclear contours, chromatin clumps, swollen mitochondria (black arrow; compare with panel C), unusual membranous profiles and lipid droplets (B). Note presence of two nucleoli (A) and a meandering nucleolus (D). Original magnification A, B, D: ×3500; C: ×4000.

of change from baseline concentration in DBP-treated rabbits was not different from that of controls except at 120 min post-GnRH in the 12-week group, in which it was significantly higher ($p < 0.05$). This was probably because the baseline concentration in this exposure group was 67% lower than that of control. In adolescent exposure groups, the concentration as well as the magnitude of change in testosterone at 30 and 120 min post-GnRH was significantly lower ($p < 0.05$) in DBP-treated rabbits at 12 weeks; this effect was not apparent at 25 weeks. In postpubertal exposure, the response of serum testosterone to exogenous GnRH was similar between controls and DBP-treated rabbits.

DISCUSSION

DBP caused developmental and/or reproductive toxicity in male Dutch-Belted rabbits exposed *in utero*, during adolescence, or after puberty. The most pronounced effects were observed in rabbits exposed *in utero*. This supports earlier findings in rodents that the male reproductive system is more susceptible to DBP (Mylchreest *et al*., 1998, 1999, 2000; Wine *et al*., 1997) or diethylhexyl phthalate (DEHP; Gray *et al*., 2000; Parks *et al*., 2000) in developing animals than in adult animals.

Rats exposed to >500 mg DBP/kg/day from GD 12 to 21 had a high incidence of testicular maldescent and impaired differentiation of Wolffian duct (epididymis and seminal vesicle) and urogenital sinus/genital tubercle (prostate and external genitalia) (Mylchreest *et al*., 1999, 2000). Reflective of these perturbations in androgen-dependent phenomena, the weights of the testes, epididymides, seminal vesicles, and prostate were also reduced at PND 100, long after cessation of treatment (Mylchreest *et al.*, 1999). Similarly, we observed cryptorchidism and malformations of reproductive tract (1/17) and decreased weights of the testes (12 weeks) and accessory glands (12 and 25 weeks) in rabbits exposed to DBP from GD 15 to 29. One of the 11 rabbits exposed during adolescence also had testicular maldescent and decreased weights of acces-

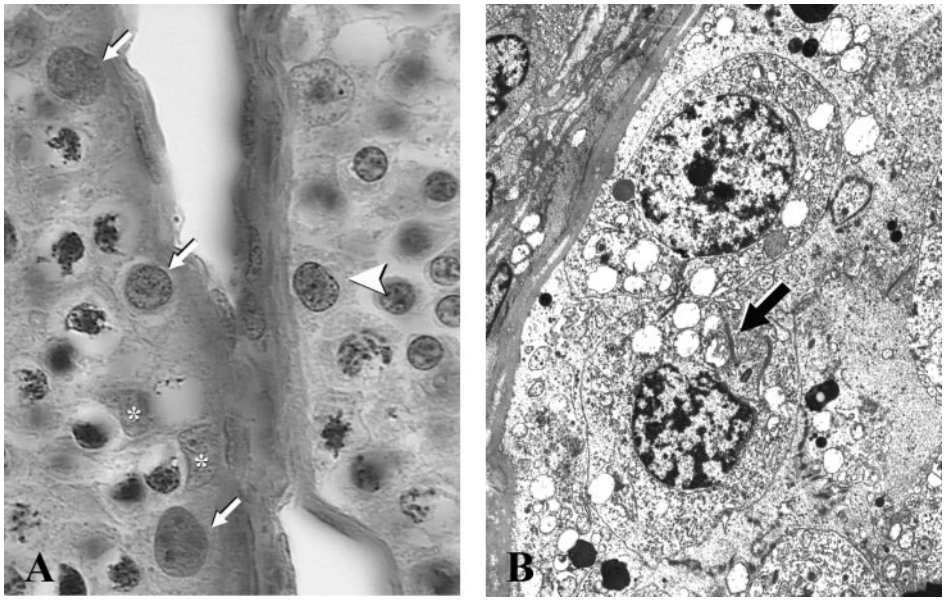

FIG. 6. Photomicrographs of atypical germ cells from a male rabbit exposed to DBP after puberty. Light microscopic examination of H&E-stained sections (A) revealed that these atypical germ cells (white arrows) had abnormal nuclei, perinuclear cytoplasmic inclusions, and pale cytoplasm compared to a normal germ cell (arrowhead). In addition, upon electron microscopic examination (B), abnormal nucleoli, irregular nuclear contours, chromatin clumping and mitochondrial swelling (black arrow) were observed. Asterisks indicate Sertoli cell nuclei. Original magnification A: ×1125; B: ×3500.

sory sex glands at 12 weeks. The low incidences of the reproductive tract malformations observed in rabbits compared to those observed in rodent studies may be due to a lower dose (400 mg DBP/kg/day in rabbit vs. >500 mg DBP/kg/day in rodents; Mylchreest *et al*., 2000) or species differences in metabolism of DBP (Foster *et al.*, 1983). Despite the low incidence, these findings confirm the previously reported adverse effects of DBP on the developing male reproductive system.

In rodents, *in utero* exposure to DBP also caused Leydig cell hyperplasia, testicular atrophy with widespread germ cell loss, multinuclear gonocytes, and decreased number of homogenization-resistant spermatids (Mylchreest *et al.*, 1998, 2002; Wine *et al*., 1997). Similarly, rodents treated with 1000 mg DEHP/kg/day from PND 6 to 11 had decreased proliferation of Sertoli cells immediately after the final dose, which eventually recovered to normal levels by PNW 6. In spite of the recovery in number of Sertoli cells, a decrease in the number of testicular spermatids was observed at PNW 19 (Dostal *et al.*, 1988). It was not clear how these perturbations in rat testis would have ultimately manifested in its final product—the mature spermatozoa.

By using the rabbit as an animal model, we were able to overcome the inherent limitations of use of rats in which collection and evaluation of seminal ejaculates are not easily accomplished and some aspects of sexual differentiation are neonatal rather than prenatal. We observed deteriorated seminal parameters in rabbits exposed to DBP *in utero* (25 weeks after the cessation of treatment). *In utero* exposure caused a reduction in ejaculate volume whereas adolescent and postpubertal exposures did not, indicating a permanent effect on function of accessory sex glands of rabbits exposed to DBP *in utero.* Sperm concentration was also lower following *in utero* exposure but not after adolescent or postpubertal exposure to DBP. Considering that both daily sperm production and epididymal sperm reserves were unaffected following *in utero* exposure, the lower sperm concentration in this group suggests impairment in epididymal transit and/or emission of sperm during ejaculation.

Irrespective of the window of exposure, DBP treatment lowered the number of morphologically normal sperm in the ejaculates, although the effect was more robust after *in utero* exposure. The majority of the abnormal sperm, regardless of window of exposure, included acrosomal and nuclear defects. Morphogenesis of these defects was observed in later stages of seminiferous epithelial cycle indicating that they originated during spermiogenesis and not during epididymal transit. Furthermore, the fact that these defects continued to occur 25 weeks and 13 weeks after the cessation of treatment (for *in utero* and adolescent exposures, respectively) indicated that these effects are lasting.

In addition, we observed atypical germ cells resembling carcinoma *in situ* (CIS) in the undescended testes of one rabbit exposed to DBP *in utero* and another treated during adolescence. CIS is believed to arise from transformation of gonocytes or pre-spermatogonia, and it is generally accepted that testicular germ cell tumors, except for spermatocytic seminoma, develop from CIS (Diekmann and Skakkebaek, 1999; Looijenga and Oosterhuis, 1999). It has been suggested that CIS cells also occur in the infantile testis adjacent to teratomas (yolk sac tumors). Since transformation of germ cells in the rabbit testis to CIS-like cells seems to be associated with the type of chemical insult and not with the abdominal location of the testis per se (Veeramachaneni *et al.*, 2001b), it is logical to conclude that DBP induces unique testicular lesions in the rabbit. CIS-like cells have been observed directly adjacent to a

TABLE 4

Effects of *in Utero,* Adolescent or Postpubertal Exposures to 400 mg DBP/kg/day on Reproductive Hormones

	12 wk			25 wk			Postpubertal exposure			
							Control		DBP	
	Control	*In utero*	Adolescent	Control	*In utero*	Adolescent	Before treatment	After treatment	Before treatment	After treatment
Hypothalamic GnRH (ng)	9.15 ± 2.20 (6)	11.08 ± 2.13 (6)	17.62 ± 1.58* (5)	11.82 ± 1.28 (6)	10.07 ± 0.93 (6)	11.23 ± 2.24 (6)	—	13.97 ± 2.57 (6)	—	13.25 ± 1.25 (6)
Serum testosterone (ng/ml)										
Pre-GnRH	6.21 ± 2.29 (6)	1.39 ± 0.48 (5)	8.06 ± 3.21 (5)	6.10 ± 2.31 (6)	3.07 ± 1.01 (6)	1.35 ± 0.38 (6)	2.44 ± 0.72 (6)	0.64 ± 0.09* (6)	2.35 ± 1.44 (6)	2.65 ± 0.83 (6)
30 min post-GnRH	18.23 ± 1.30 (6)	22.02 ± 3.71 (5)	7.80 ± 0.95** (5)	14.36 ± 0.70 (6)	16.49 ± 1.60 (6)	10.40 ± 2.74 (6)	—	13.85 ± 4.77 (6)	—	10.17 ± 0.80 (6)
Change[a]	12.02 ± 2.35 (6)	20.63 ± 4.01 (5)	−0.27 ± 2.43** (5)	8.26 ± 2.02 (6)	13.42 ± 2.30 (6)	9.05 ± 2.54 (6)	—	13.21 ± 4.80 (6)	—	7.52 ± 1.39 (6)
120 min post-GnRH	16.52 ± 0.98 (6)	21.82 ± 3.34 (5)	10.57 ± 2.08** (5)	13.74 ± 2.19 (4)	17.11 ± 3.65 (6)	8.25 ± 2.53 (6)	—	8.90 ± 2.66 (6)	—	9.60 ± 0.84 (6)
Change[a]	10.31 ± 2.50 (6)	20.43 ± 3.65* (5)	2.51 ± 2.03* (5)	10.89 ± 2.02 (4)	14.04 ± 3.98 (6)	6.90 ± 2.35 (6)	—	8.26 ± 2.59 (6)	—	6.96 ± 0.91 (6)

Note. Values represent mean ± SEM. Numbers in parentheses indicate number of animals.

[a] Change from baseline serum testosterone concentration; because of unsuccessful blood collection at stipulated time points, values for post-GnRH challenge are missing in some instances.

* $p < 0.05$ and ** $p < 0.01$ using t-test.

spontaneously occurring seminoma-like tumor in this species (Veeramachaneni and VandeWoude, 1999). In our studies with Dutch-Belted rabbits we have never observed abdominal testes, malformed accessory sex glands, or feminized external genitalia in an untreated male. To date, out of 114 control male rabbits (in our 6 historical studies) we have encountered only one animal with a testis located in the inguinal canal. We have seen CIS-like cells in the testes of other treated male offspring, which also were associated with cryptorchidism (Veeramachaneni, 2000a,b; Veeramachaneni *et al*., 2001b).

We also observed atypical germ cells in one rabbit exposed to DBP after puberty. It is assumed that these atypical cells were derived from a more differentiated phenotype than a gonocyte (Looijenga and Oosterhuis, 1999), since exposure began well beyond infantile and pubertal development when gonocytes are not expected to be present. As with the CIS-like cells observed in *in utero* and adolescent exposures, the fate of atypical germ cells observed in postpubertal exposure is not known. It is interesting to note that spermatocytic seminoma (a form of testicular cancer that occurs in older men) develops from transformed B spermatogonia but not from CIS (Dekker *et al.*, 1992). Detailed morphometric analysis of the incidence of the atypical germ cells observed in DBP-treated rabbits and studies investigating whether these cells ultimately develop into cancerous lesions are warranted.

All exposures in the current study caused germinal epithelial loss in DBP-treated rabbits as indicated by a slight but significant increase in DGEL immediately following or several months after cessation of treatment. These observations are consistent with the reports of increased germ cell loss in rodents without recovery as adults (Mylchreest *et al.*, 1998, 1999). Likewise, acute exposure of postpubertal rodents to high doses of DBP (2000–2400 mg DBP/kg/day) has been reported to cause severe testicular atrophy and immediate reduction in testis weight (Cater *et al.*, 1977; Foster *et al.*, 1980; Gray *et al.*, 1982).

Phthalate-mediated male reproductive toxicity in the developing testis is thought to occur by a complex interaction between Sertoli, Leydig, and germ cells (Mylchreest *et al.*, 2002). In the current study, *in utero* or adolescent exposure to DBP decreased concentrations of testosterone at 6weeks. In rats, *in utero* phthalate treatment alters Leydig cell differentiation such that the cells proliferate, forming large clusters, rather than differentiating. The resultant immature fetal Leydig cells fail to synthesize normal testosterone and insl-3 (Wilson, Gray *et al*., in preparation). During puberty, phthalates appear to inhibit FSH-mediated processes in Sertoli cells, and increase the exfoliation of germ cells and incidence of multinuclear gonocytes (Li *et al.*, 1998, 2000). Furthermore DBP decreases the testicular testosterone concentrations by inhibiting 3β-hydroxysteroid dehydrogenase, a key enzyme in the steroidogenic pathway (Mylchreest *et al.*, 2002); however, it is unclear if this represents a direct effect of phthalate monoesters on this enzyme.

In utero exposure to DBP did not alter the hypothalamic GnRH content or the basal or GnRH-stimulated concentrations of testosterone either at 12 or 25 weeks. It should be noted, however, that although rabbits exposed to DBP during adolescence had basal levels of testosterone similar to controls at 12 weeks, GnRH-stimulated testosterone production was significantly decreased 30 or 120 min post-stimulation. Furthermore, the hypothalamic GnRH content was higher in DBP-treated rabbits at 12 weeks. Without measuring the concentrations of gonadotropins, we were unable to ascertain whether this effect was mediated by lowered pituitary responsiveness to GnRH or the inability of the testis to respond to gonadotropin. This effect was not permanent, and the ability to respond to GnRH-stimulation returned to normal at 25 weeks. Comparable effects were found in Leydig cell steroidogenic function of rats exposed to DEHP (Akingbemi *et al.,* 2001). Thus, similar to observations made in rodents, it appears that *in utero* exposure to DBP directly affects the endocrine milieu of the developing rabbit testis, probably by acting on differentiating Leydig cells.

It is uncertain if the phthalate-induced testicular toxicity in postpubertal rodents was a result of changes in gonadotropins and gonadal hormones or a direct effect on the testis. A majority of the studies using rats indicates testicular toxicity during this life stage is a result of compromised Sertoli cell function, since metabolic and morphological changes are observed immediately after treatment (Creasy *et al.*, 1983, 1987; Foster *et al.*, 1980; Gray and Gangolli, 1986; Heindel and Chapin, 1989; Heindel and Powell, 1992). Because hypothalamic GnRH content and concentrations of testosterone were not affected following DBP treatment in postpubertal rabbits, we assume DBP-induced testicular changes occurred without altering the hormones necessary to drive reproductive or metabolic processes, supporting the hypothesis of a direct effect on Sertoli cells or germ cells.

Mating ability was not affected to any significant extent in any exposure window. Similarly, mating ability was not affected in rats exposed to 80 or 385 mg DBP/kg/day from GD 0 to PND 21 (Wine *et al.*, 1997). Since sexual function in rabbits is known to be sensitive to developmental exposure of a variety of xenobiotics (Veeramachaneni *et al*., 2000a,b, Veeramachaneni *et al*., 2001a), lack of any significant detrimental effect on mating ability following DBP exposure indicates that this chemical does not affect sexual function.

The observations that *in utero*, adolescent, or postpubertal exposure to DBP decreased the percentage of morphologically normal sperm in the ejaculate and increased germinal epithelial loss, and that exposure to DBP *in utero* or during adolescence decreased the number of sperm in the ejaculate by 23 and 20% should not be overlooked. Human males have low reproductive efficiency, only ejaculating twice the number of normal sperm where fertility might be expected to decline (Amann, 1982). Therefore, the human testis would be more vulnerable to toxicological insult, assuming similar responses occur. Several epidemiological studies have attempted to correlate exposure

to phthalate-containing materials with decline in semen quality and increased risk of testicular cancer (Murature *et al.*, 1987; Hardell *et al.*, 1997; Hansen, 1999). A weak negative correlation between sperm density and phthalate concentration was found in a random population of college students (Murature *et al.*, 1987), while a 7-fold increase in risk for developing testicular cancer was determined in workers occupationally exposed to phthalate-containing materials (Hardell *et al.*, 1997). However, another study using a larger population found no increased risk for testicular cancer in these workers (Hansen, 1999). More recently, Duty *et al*., (2002) reported that urinary DEP levels were associated with increased DNA damage in human sperm.

Collectively, these results indicate that exposure to DBP during development affects the male rabbit, a nonrodent species, and that males exposed to DBP *in utero* or during adolescence are more sensitive to toxicological injury to the reproductive system than adults.

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